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CLASS 426

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SUBCLASS 049

GROUP ART UNIT 1302

EXAMINER Wong

APPLICANTS JED W. FAHEY, ELDERSBURG, MD; PAUL TALALAY, BALTIMORE, MD.

\*\*CONTINUING DATA\*\*\*\*\*  
VERIFIED

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\*\*FOREIGN/PCT APPLICATIONS\*\*\*\*\*  
VERIFIED

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FOREIGN FILING LICENSE GRANTED 12/19/95 \*\*\*\*\* SMALL ENTITY \*\*\*\*\*

Foreign priority claimed 35 USC 119 conditions met	<input type="checkbox"/> yes <input type="checkbox"/> no	<input type="checkbox"/> yes <input type="checkbox"/> no	AS FILED	STATE OR COUNTRY	SHEETS DRWGS.	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED	ATTORNEY'S DOCKET NO.
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ADDRESS FOLEY AND LARDNER  
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SUITE 500  
WASHINGTON DC 20007-5109

TITLE METHOD OF PREPARING A FOOD PRODUCT FROM CRUCIFEROUS SEEDS

U.S. DEPT. OF COMM./ PAT. & TM—PTO-436L (Rev. 12-94)

PARTS OF APPLICATION  
FILED SEPARATELY

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8-14-97

Assistant Examiner

CLAIMS ALLOWED

Total Claims 16

Print Claim 1

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LESLIE WONG  
PRIMARY EXAMINER  
GROUP 1300

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Figs. Drwg. 23

Print Fig. None

ISSUE  
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NUMBER 043

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Leslie Wong 8/13/97 Primary Examiner

PREPARED FOR ISSUE

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08 528858

PATENT APPLICATION



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## CONTENTS

Date  
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	1. Application <u>2 draw</u> papers.	
	2. <u>Get Small Entity</u>	10-24-95
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POSITION	ID NO.	DATE
CLASSIFIER	21	10/26/95
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## INDEX OF CLAIMS

Claim	Final	Original	Date
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## SYMBOLS

✓ ..... Rejected  
 = ..... Allowed  
 - (Through numeral) ..... Canceled  
 + ..... Restricted  
 N ..... Non-elected  
 I ..... Interference  
 A ..... Appeal  
 O ..... Objected

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**SEARCHED**

Class	Sub.	Date	Exmr.
424	049	12/19/96	L.Wmy
426	007 044 049 052 615 629 655 425 429 430 431	5/22/97	L.Wmy
426	007 044 049 052 615 629 655 425 429 430 431	8/12/97	L.Wmy


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**SEARCH NOTES**

	Date	Exmr.
MS	12/19/96	L.Wmy

**INTERFERENCE SEARCHED**

Class	Sub.	Date	Exmr.
426	049 052 615 425 429 431	8/13/97	L.Wmy

BAR CODE LABEL						U.S. PATENT APPLICATION	
SERIAL NUMBER		FILING DATE		CLASS	GROUP ART UNIT		
08/528,858		09/15/95		426	1302		
APPLICANT	JED W. FAHEY, ELDERSBURG, MD; PAUL TALALAY, BALTIMORE, MD.  **CONTINUING DATA***** VERIFIED _____  **FOREIGN/PCT APPLICATIONS***** VERIFIED _____  FOREIGN FILING LICENSE GRANTED 12/19/95						
STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS	FILING FEE RECEIVED	ATTORNEY DOCKET NO.		
MD	2	47	13	\$2,084.00	46528/102/JO		
ADDRESS	FOLEY AND LARDNER 3000 K STREET NW SUITE 500 WASHINGTON DC 20007-5109						
TITLE	CANCER CHEMOPROTECTIVE FOOD PRODUCTS						
This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application which is identified above.  By authority of the COMMISSIONER OF PATENTS AND TRADEMARKS							
Date	1-B5 Certifying Officer						



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08 528858

Assistant Commissioner for Patents  
Washington, D. C. 20231

Sir:

Transmitted herewith for filing is the patent application of:

INVENTOR(S): **Jed W. FAHEY, Paul TALALAY**

TITLE: **CANCER CHEMOPROTECTIVE FOOD PRODUCTS**

In connection with this application, the following are enclosed:

- 44 Pages of Specification with Abstract
- 47 Claims
- 2 Sheets of Drawings
- XX Declaration, Power of Attorney
- XX Assignment to: Johns Hopkins School of Medicine
- Certified Priority Application and Priority Claim
- Statement of Small Entity Status
- Other:

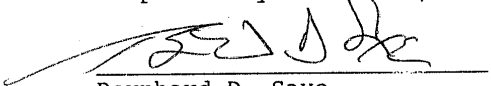
The fee has been calculated as shown below. (Small entity fees indicated in parentheses.)

(1) For	(2) Number Filed	(3) Number Extra	(4) Rate	(5) Basic Fee \$730 (\$365)
Total Claims	47 - 20 =	27	x \$22 (x \$11)	594.00
Independent Claims	14 - 3 =	11	x \$76 (x \$38)	836.00
Multiple Dependent Claims			\$240 (\$120)	-
Assignment Recording Fee			\$ 40	40.00
TOTAL FEE:				\$2,200.00

A check in the amount of the above TOTAL FEE is attached. This amount is believed to be correct; however, the Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. 19-0741.


Respectfully submitted,

Date: September 15, 1995  
Docket No.: 46528/102/JOHO

  
Bernhard D. Saxe  
Req. No. 28,665



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REF - 40906 SEP 15 1995		PAY TO THE ORDER OF COMMISSIONER OF PATENTS & TRADEMARKS 46528102	
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September 15, 1995

The "Received" stamp of the U.S. Patent & Trademark Office hereon acknowledges receipt of the accompanying Patent Application of FAHEY *et al.*

for CANCER CHEMOPROTECTIVE FOOD PRODUCTS

Including:

44 pages of spec.; 47 claims; 2 shs. dws. XX Declaration; XX Oath; XX Assignment; and Check for \$2,200.00

Filed September 15, 1995



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PATENT AND TRADEMARK OFFICE  
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19-0741 180 101 76.00CR

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Inventors: Jed W. Fahey  
Paul Talalay

METHOD OF PREPARING A FOOD PRODUCT FROM  
CRUCIFEROUS SEEDS  
~~CANCER-CHEMOPROTECTIVE FOOD PRODUCTS~~

The U.S. Government has a paid-up license in this  
5 invention and the right in limited circumstances to  
require the patent owner to license others on reasonable  
terms as provided for by the terms of grant PO1 CA 44530,  
entitled "Novel Strategies for Chemoprotection Against  
Cancer", (Paul Talalay, Principal Investigator) awarded  
10 by the National Cancer Institute, Department of Health  
and Human Services.

**BACKGROUND OF THE INVENTION**

**I. Field of Invention**

This invention relates to a dietary approach to  
15 reducing the level of carcinogens in animals and their  
cells and thereby reducing the risk of developing cancer.  
In particular, this invention relates to the production  
and consumption of foods which are rich in cancer  
chemoprotective compounds. More specifically, this  
20 invention relates to chemoprotective compounds that  
modulate mammalian enzymes which are involved in  
metabolism of carcinogens. This invention relates to  
food sources which are extremely rich in compounds that  
induce the activity of Phase 2 enzymes, without inducing  
25 biologically significant activities of those Phase 1  
enzymes that activate carcinogens.

**II. Background**

It is widely recognized that diet plays a large role  
in controlling the risk of developing cancers and that  
30 increased consumption of fruits and vegetables reduces  
cancer incidence in humans. It is believed that a major  
mechanism of protection depends on the presence of  
chemical components in plants that, when delivered to

mammalian cells, elevate levels of Phase 2 enzymes that detoxify carcinogens.

Early studies on the mechanism of chemoprotection by certain chemicals assumed that these chemoprotectors induced activities of monooxygenases, also known as Phase 1 enzymes or cytochromes P-450. However, Talalay *et al.*, [reviewed in "Chemical Protection Against Cancer by Induction of Electrophile Detoxication (Phase II) Enzymes" In: CELLULAR AND MOLECULAR TARGETS OF CHEMOPREVENTION, L. Wattenberg *et al.*, CRC Press, Boca Raton, FL, pp 469-478 (1992)] determined that administration of the known chemoprotector butylated hydroxyanisole (BHA) to rodents resulted in little change in cytochromes P-450 (Phase 1 enzyme) activities, but profoundly elevated Phase 2 enzymes. Phase 2 enzymes such as glutathione transferases, NAD(P)H:quinone reductase (QR) and glucuronosyltransferases, detoxify DNA-damaging electrophilic forms of ultimate carcinogens. Selective inducers of Phase 2 enzymes are designated monofunctional inducers. Prochaska & Talalay, *Cancer Res.* 48: 4776-4782 (1988). The monofunctional inducers are nearly all electrophiles and belong to 8 distinct chemical classes including (1) diphenols, phenylenediamines and quinones; (2) Michael reaction acceptors containing olefins or acetylenes conjugated to electron-withdrawing groups; (3) isothiocyanates; (4) 1,2-dithiole-3-thiones; (5) hydroperoxides; (6) trivalent inorganic and organic arsenic derivatives; (7) heavy metals with potencies related to their affinities for thiol groups including  $Hg^{2+}$ , and  $Cd^{2+}$ ; and (8) vicinal dimercaptans. Prester *et al.*, *Proc. Natl. Acad. Sci. USA* 90: 2963-2969 (1993). The only apparent common property shared by all of these inducers is their ability to react with thiol groups.

Chemoprotective agents can be used to reduce the susceptibility of mammals to the toxic and neoplastic effects of carcinogens. These chemoprotectors can be of

plant origin or synthetic compounds. Synthetic analogs of naturally occurring inducers have also been generated and shown to block chemical carcinogenesis in animals. Posner et al., *J. Med. Chem.* 37: 170-176 (1994); Zhang et al., *Proc. Natl. Acad. Sci. USA* 91: 3147-3150 (1994);  
5 Zhang et al., *Cancer Res. (Suppl)* 54: 1976s-1981s (1994).

Highly efficient methods have been developed for measuring the potency of plant extracts to increase or induce the activities of Phase 2 enzymes. Prochaska &  
10 Santamaria, *Anal. Biochem.* 169: 328-336 (1988) and Prochaska et al., *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992). In addition, these methods have been employed for isolating the compounds responsible for the inducer activities in plants and for evaluating the  
15 anticarcinogenic activities of these compounds and their synthetic analogs. Zhang et al., *Proc. Natl. Acad. Sci. USA* 89: 2399-2403 (1992) and Posner et al., *J. Med. Chem.* 17: 170-176 (1994).

Although inducer activity has been found in many  
20 different families of edible plants, the amounts are highly variable, depending on family, genus, species, variety, or cultivar of the plant selection and on growth and harvesting conditions. Thus, there is a need in the art to identify particular edible plants and methods of  
25 growing and preparing them that yield high levels of Phase 2 enzyme-inducer activity for chemoprotection. There is also a need for methods of growing and preparing edible plants that produce a known spectrum of specific inducers of Phase 2 enzyme activity in order to increase  
30 the efficiency with which specific carcinogens, or classes of carcinogens, are targeted for inactivation. In addition, there is a need for methods of plant breeding and selection to increase the level of Phase 2 inducer activity and to manipulate the spectrum of  
35 inducers produced in particular cultivars.

*SUMMARY OF THE INVENTION*

It is an object of the present invention to provide food products and food additives that are rich in cancer chemoprotective compounds.

5 Another object of the present invention is to provide food products which contain substantial quantities of Phase 2 enzyme-inducers and are essentially free of Phase 1 enzyme-inducers.

10 It is a further object of the present invention to provide food products which contain substantial quantities of Phase 2 enzyme-inducing potential and non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

15 These objects, and others, are achieved by providing cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage. The cruciferous sprouts include *Brassica oleracea* varieties *acephala*, *alboglabra*, *botrytis*, *costata*, *gemmifera*, *gongylodes*, *italica*, *medullosa*,  
20 *palmifolia*, *ramosa*, *sabauda*, *sabellica*, and *selensia*.

Another embodiment of the present invention provides cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage, wherein the sprouts are substantially free  
25 of Phase 1 enzyme-inducing potential.

Yet another embodiment of the present invention provides a non-toxic solvent extract of cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage.  
30 The non-toxic solvent extract can be a water extract. In addition, the water extract can comprise a cruciferous vegetable, such as a cruciferous vegetable of the genus *Raphanus*, comprising an active myrosinase enzyme.



Another embodiment of the present invention provides a food product comprising cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage; extracts of the sprouts or cruciferous seeds; or any combination of the sprouts or extracts.

A further embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage.

Yet another embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of a food product comprising cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage.

Another embodiment of the present invention provides cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce said sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates. The cruciferous sprouts include *Brassica oleracea* varieties *acephala*, *alboglabra*, *botrytis*, *costata*, *gemmifera*, *gongylodes*, *italica*, *medullosa*, *palmifolia*, *ramosa*, *sabauda*, *sabellica*, and *selensia*.

A further embodiment of the present invention provides a food product comprising sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at

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least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days from growth of seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates; extracts of the sprouts or cruciferous seeds; or any combination of the sprouts or extracts.

Yet another embodiment of the present invention provides cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates and are substantially free of Phase 1 enzyme-inducing potential.

Another embodiment of the present invention provides a non-toxic solvent extract of cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates. The non-toxic solvent extract can be a water extract. In addition, the water extract can comprise a cruciferous vegetable, such as a cruciferous vegetable of the genus *Raphanus*, comprising an active myrosinase enzyme.

Yet another embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of cruciferous sprouts harvested prior to the 2-leaf stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when

measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

5 Yet another embodiment of the present invention provides a method of increasing the chemoprotective amount of Phase 2 enzymes in a mammal, comprising the step of administering an effective quantity of a food product comprising sprouts harvested prior to the 2-leaf  
10 stage, wherein the sprouts have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and contain non-toxic levels of indole glucosinolates and their breakdown products and  
15 goitrogenic hydroxybutenyl glucosinolates.

A further embodiment of the present invention provides a method of preparing a food product rich in glucosinolates, comprising germinating cruciferous seeds, with the exception of cabbage, cress, mustard and radish  
20 seeds, and harvesting sprouts prior to the 2-leaf stage to form a food product comprising a plurality of sprouts. The cruciferous sprouts include *Brassica oleracea* varieties *acephala*, *alboglabra*, *botrytis*, *costata*, *gemmifera*, *gongylodes*, *italica*, *medullosa*, *palmifolia*,  
25 *ramosa*, *sabauda*, *sabellica*, and *selensia* and contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

Yet another embodiment of the present invention provides a food product rich in glucosinolates made by  
30 germinating cruciferous seeds, with the exception of cabbage, cress, mustard and radish seeds, and harvesting sprouts prior to the 2-leaf stage to form a food product comprising a plurality of sprouts.

Yet another embodiment of the present invention  
35 provides a method of preparing a food product comprising

extracting glucosinolates and isothiocyanates from cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts, harvested prior to the 2-leaf stage, with a non-toxic solvent and recovering the  
5 extracted glucosinolates and isothiocyanates. Myrosinase enzyme, or a vegetable, such as *Raphanus* species, containing the enzyme is mixed with the cruciferous sprouts, the extract, or both the sprouts and the extract.

10 An embodiment of the present invention provides a method of preparing a food product rich in glucosinolates, comprising germinating cruciferous seeds having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3  
15 days of growth from seeds that produce the sprouts and which contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates, and harvesting sprouts prior to the 2-leaf stage to form a food product  
20 comprising a plurality of sprouts. The seeds may be *Brassica oleracea*, including the varieties *acephala*, *alboglabra*, *botrytis*, *costata*, *gemnifera*, *gongylodes*, *italica*, *medullosa*, *palmifolia*, *ramosa*, *sabauda*, *sabellica*, and *selensia*.

25 Yet another embodiment of the present invention provides a food product rich in glucosinolates made by germinating cruciferous seeds having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds  
30 that produce the sprouts and which contain non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates, and either harvesting sprouts at the 2-leaf stage to form a food product comprising a plurality of sprouts. The  
35 nutritional product contains non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.



A further embodiment of the present invention provides a method of preparing a food product comprising extracting glucosinolates and isothiocyanates with a solvent from cruciferous seeds, sprouts, plants or plant parts, wherein seeds that produce the sprouts, plants or plant parts producing sprouts having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth and wherein the seeds, sprouts, plants or plant parts have non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates, and recovering the extracted glucosinolates and isothiocyanates. The non-toxic extraction solvent can be water. Myrosinase enzyme, or a vegetable, such as *Raphanus* species, containing the enzyme is mixed with the cruciferous sprouts, seeds, plants, plant parts or extract, or any combination thereof.

A further embodiment of the present invention provides a method of reducing the level of carcinogens in mammals, comprising administering cruciferous sprouts, with the exception of cabbage, cress, mustard and radish sprouts.

Yet another embodiment of the present invention provides a method of reducing the level of carcinogens in mammals, comprising administering cruciferous sprouts having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when measured after 3 days of growth from seeds that produce the sprouts and non-toxic levels of indole glucosinolates and their breakdown products and goitrogenic hydroxybutenyl glucosinolates.

Another embodiment of the present invention provides a method of preparing a food product by introducing cruciferous seeds, having at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential when

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measured after 3 days of growth from seeds that produce the sprouts and non-toxic levels of indole glucosinolates and goitrogenic hydroxybutenyl glucosinolates, into an edible ingredient.

5 A further embodiment of the present invention provides a method of extracting glucosinolates and isothiocyanates from plant tissue which comprises homogenizing the plant tissue in an excess of a mixture of dimethyl sulfoxide, acetonitrile, and  
10 dimethylformamide (DMF/ACN/DMSO) at a temperature that prevents myrosinase activity.

Another embodiment of the present invention provides cruciferous sprouts harvested prior to the 2-leaf stage, wherein the ratio of monofunctional to bifunctional  
15 inducers is at least 20 to 1.

Another object of the present invention is to provide a food product supplemented with a purified or partially purified glucosinolate.

20 Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various  
25 changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### *BRIEF DESCRIPTION OF THE DRAWINGS*

30 Figure 1 shows the total inducing potential of organic solvent extracts of broccoli and daikon cultivars as a function of age.

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Figure 2 shows the high resolution NMR spectra of isolated glucosinolates obtained from hot aqueous extracts of 3-day old Saga broccoli sprouts.

#### DETAILED DESCRIPTION

##### 5 I. Definitions

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

10 A **bifunctional inducer** is a molecule which increases activities of both Phase 1 enzymes such as cytochromes P-450 and Phase 2 enzymes and requires the participation of Aryl hydrocarbon (Ah) receptor and its cognate Xenobiotic Response Element (XRE). Examples include flat planar aromatics such as polycyclic hydrocarbons, azo dyes or  
15 2,3,7,8-tetrachloro-dibenzo-p-dioxin (TCDD).

A **chemoprotector** or **chemoprotectant** is a synthetic or naturally occurring chemical agent that reduces susceptibility in a mammal to the toxic and neoplastic effects of carcinogens.

20 A **food product** is any ingestible preparation containing the sprouts of the instant invention, or extracts or preparations made from these sprouts, which are capable of delivering Phase 2 inducers to the mammal ingesting the food product. The food product can be  
25 freshly prepared such as salads, drinks or sandwiches containing sprouts of the instant invention. Alternatively, the food product containing sprouts of the instant invention can be dried, cooked, boiled, lyophilized or baked. Breads, teas, soups, cereals,  
30 pills and tablets, are among the vast number of different food products contemplated.

**Inducer activity** or **Phase 2 enzyme-inducing activity** is a measure of the ability of a compound(s) to induce

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Phase 2 enzyme activity. In the present invention, inducer activity is measured by means of the murine hepatoma cell bioassay of QR activity *in vitro*. Inducer activity is defined herein as QR inducing activity in Hepa 1c1c7 cells (murine hepatoma cells) incubated with extracts of sprouts, seeds or other plant parts untreated with myrosinase. Inducer activity is measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates. Typically 10,000 Hepa 1c1c7 cells are introduced into each well. Hepatoma cells are grown for 24 hours and a plant extract containing microgram quantities of fresh plant tissue is serially diluted across the microtiter plates into fresh culture medium containing 0.15 ml  $\alpha$ MEM culture medium amended with 10% Fetal Calf Serum (FCS) and streptomycin and penicillin. The cells are further incubated for 48 hours. QR activity (based on the formation of the blue-brown reduced tetrazolium dye) is measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and related to its protein concentration. Quantitative information on specific activity of QR is obtained by computer analysis of the absorbances. One unit of inducer activity is the amount that when added to a single microtiter well doubles the QR activity. (See Prochaska and Santamaria, *Anal. Biochem.* 169: 328-336 (1988) and Prochaska et al., *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992)).

Inducer potential or Phase 2 enzyme-inducing potential is a measure of the combined amounts of inducer activity in plant tissue provided by isothiocyanates, plus glucosinolates that can be converted by myrosinase to isothiocyanates. Glucosinolates are not themselves inducers of mammalian Phase 2 enzymes, whereas isothiocyanates are inducers. Inducer potential therefore is defined herein as QR activity in murine 1c1c7 hepatoma cells incubated with myrosinase-treated extracts of the sprouts, seeds or other plant parts. In the present invention therefore inducer potential is measured by means of the murine hepatoma cell bioassay of



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QR activity *in vitro* as described above. Inducer potential is measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates. Typically, 10,000 Hepa 1c1c7 cells are introduced into each well. Hepatoma  
5 cells are grown for 24 hours and a plant extract containing microgram quantities of fresh plant tissue is serially diluted across the microtiter plates into fresh culture medium containing 0.15 ml  $\alpha$ MEM culture medium amended with 10% Fetal Calf Serum (FCS) and streptomycin  
10 and penicillin. Myrosinase (6 units/ml plant extract) is added to the plant extract. Myrosinase is purified by modification of the technique of Palmieri *et al.*, *Anal. Biochem.* 35: 320-324 (1982) from 7 day old Daikon sprouts grown on agar support containing no added nutrients.  
15 Following 234-fold purification, the myrosinase had a specific activity of 64 units/mg protein [unit = amount of enzyme required to hydrolyze 1  $\mu$ mol sinigrin/min]. Plant extract is diluted 200-fold into the initial wells of the microtiter plate followed by 7 serial dilutions.  
20 The cells are further incubated for 48 hours. QR activity (based on the formation of the blue-brown reduced tetrazolium dye) is measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and related to its protein concentration.  
25 Quantitative information on specific activity of QR is obtained by computer analysis of absorbances. One unit of inducer potential is the amount that when added to a single microtiter well doubles the QR activity. (See Prochaska and Santamaria, *Anal. Biochem.* 169: 328-336 (1988) and Prochaska *et al.*, *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992)).  
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A monofunctional inducer increases the activity of Phase 2 enzymes selectively without significantly altering Phase 1 enzyme activities. Monofunctional  
35 inducers do not depend on a functional Ah receptor but enhance transcription of Phase 2 enzymes by means of an Antioxidant Responsive Element (ARE).

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A cruciferous sprout is a plant or seedling that is at an early stage of development following seed germination. Cruciferous seeds are placed in an environment in which they germinate and grow. The cruciferous sprouts of the instant invention are harvested following seed germination through and including the 2-leaf stage. The cruciferous sprouts of instant invention have at least 200,000 units per gram fresh weight of Phase 2 enzyme-inducing potential at 3-days following incubation under conditions in which cruciferous seeds germinate and grow.

## II. Description

A major mechanism of protection provided by fruits and vegetables in reducing the cancer incidence in humans depends on minor chemical components which, when delivered to mammalian cells, elevate levels of Phase 2 enzymes that detoxify carcinogens. It has now been discovered that the anticarcinogenic activity of certain edible plants can be increased. Plants such as *Brassica oleracea* variety *italica* (broccoli) are normally not harvested until they form heads. By growing these plants only to the seedling or sprout stage, that is between the onset of germination and the 2-leaf stage, the levels of inducers of enzymes that detoxify carcinogens and protect against cancer can be increased at least five-fold over those found in commercial stage vegetables of the same cultivars. Often increases of between 10 and 1000-fold have been observed.

Harvesting plants at an early seedling or sprout stage, or otherwise arresting their growth, leads to the greatest inducer potential and yields a food product of a type to which consumers are already accustomed. The Phase 2 enzyme-inducing potential of such sprouts may be as much as several hundred times higher than that observed in adult, market stage vegetables obtained from the same seeds. Thus it is possible that humans can consume the same quantities of inducer potential by

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eating relatively small quantities of sprouts, rather than large quantities of market-stage vegetables.

It has now been found that most of the inducer potential of crucifer plants is due to their content of isothiocyanates and their biogenic precursors, glucosinolates. Glucosinolates are converted to isothiocyanates by the enzyme myrosinase which is a thioglucosidase. Normally myrosinase and glucosinolates are separated in the cell and if the cell is damaged, with loss of compartmentalization, myrosinase comes into contact with glucosinolates, which are then converted to isothiocyanates.

In order to screen large numbers of edible plants and to evaluate the effects of environmental perturbation on Phase 2 enzyme-inducer potential in those vegetables, it was necessary to improve upon the previously described techniques for homogenization and extraction of those vegetables. Techniques initially described for the extraction of Phase 2 inducers from vegetables involved homogenization of the vegetables in cold water, lyophilization, extraction of the resultant powder with acetonitrile, filtration and evaporative concentration, Prochaska et al., *Proc. Natl. Acad. Sci. USA* 89: 2394-2398 (1992).

Following identification of sulforaphane as the principal Phase 2 inducer from broccoli, comparative extractions were performed into hot 80% methanol, yielding similar inducer activity as the aforementioned acetonitrile extracts. When myrosinase was added to these hot methanol extracts in which glucosinolates are freely soluble, there was a dramatic enhancement of the Phase 2 inducer activity of these extracts (data summarized in Table 1). The deliberate conversion of these glucosinolates to isothiocyanates using exogenous myrosinase thus gave a better index of the inducers for Phase 2 enzymes of the vegetables tested. It was thus

clear that the majority of the potential Phase 2 inducers in crucifers was usually present in whole plants as the glucosinolate precursors of isothiocyanates.

5       The preponderance of glucosinolates and the rapidity  
with which, upon wounding of cruciferous plant tissue,  
glucosinolates are converted to isothiocyanates, led to  
the development of an improved extraction procedure. By  
manipulation of solvent mixtures and of the water  
10       activity of fresh vegetable/solvent homogenates, a  
procedure was developed that permits both glucosinolate  
and isothiocyanate quantification from the same,  
non-concentrated sample. In addition to being the  
rate-limiting step in an extraction protocol, evaporative  
15       concentration allows volatile inducers to escape  
detection. The improved procedure is both simple and  
efficient, requiring only that the plant sample be  
completely homogenized in solvent. Using this technique,  
the present inventors have thus been able to demonstrate  
20       dramatic increases in the recovery of inducer activity  
and inducer potential from cruciferous vegetables over  
previously described techniques.

      If fresh-picked vegetables are promptly and gently  
harvested, directly into organic solvents comprising a  
mixture of DMF/ACN/DMSO and a temperature that prevents  
25       myrosinase activity, both glucosinolates and  
isothiocyanates are efficiently extracted into the  
organic solvent mixture. Preferably, the DMF, ACN and  
DMSO are mixed in equal volumes. However, the volumes of  
the three solvents in the mixture can be varied to  
30       optimize extraction of specific glucosinolates and  
isothiocyanates from any plant tissue. The temperature  
of the extraction mixture is preferably less than 0°C,  
and most preferably less than -50°C. The temperature of  
the extraction solvent must be kept above freezing. At  
35       the same time the enzyme myrosinase, which invariably  
accompanies these constituents in the plants and rapidly  
converts glucosinolates into isothiocyanates, is

inactive. Such extracts typically contain high quantities of glucosinolates and negligible quantities of isothiocyanates. The *in planta* myrosinase activity varies between different plant species.

5        Glucosinolates are not themselves inducers of mammalian Phase 2 enzymes, whereas isothiocyanates are monofunctional inducers in the murine hepatoma cell bioassay of QR activity. The inducer potential, as distinct from inducer activity, of plant extracts can be  
10        measured by adding purified myrosinase, obtained from the same, or other plant sources, to the assay system.

      Glucosinolates are converted at least partially to isothiocyanates in humans. If, however, it is desirable to accelerate this conversion, broccoli or other  
15        vegetable sprouts, high in glucosinolates, can be mixed with myrosinase. The mixture can be in water, or some other non-toxic solvent that does not inactivate myrosinase. The myrosinase can be from a partially purified or purified preparation. Alternatively, the  
20        myrosinase can be present in plant tissue, such as a small quantity of crucifer sprouts rich in myrosinase, including *Raphanus sativus* or daikon. Such a preparation can be used to produce a "soup" for ingestion that is high in isothiocyanates and low in glucosinolates.  
25        Inducer potential can be measured using a multiwell plate screen with murine hepatoma cells for *in vitro* measurement of QR specific activity as described above.

      The ratio of monofunctional to bifunctional inducer activity of plant tissue is measured by bioassaying plant  
30        extracts, as described above, not only in wild-type Hepa 1c1c7 cells, but also, in mutants designated c1 and BP'c1 that have either defective Ah receptors or defective cytochrome P<sub>1</sub>-450 genes, respectively. Prochaska and Talalay, *Cancer Research* 48: 4776-4782 (1988).